Please note: the shared protocols described herein may not have been validated by Pacific Biosciences and are provided as-is and without any warranty. Use of these protocols is offered to those customers who understand and accept the associated terms and conditions and wish to take advantage of their potential to help prepare samples for analysis using the PacBio® System. If any of these protocols are to be used in a production environment, it is the responsibility of the end user to perform the required validation.

Preparing >30 kb SMRTbell™ Libraries Using Needle Shearing and BluePippin™ Size-Selection System

Before You Begin

This document provides recommendations for preparing >30 kb size-selected SMRTbell libraries from 5 µg of starting sheared genomic DNA (gDNA).

Only high-quality, high molecular weight gDNA may be used for producing >30 kb libraries. To ensure success, gDNA size and integrity must be verified by pulsed field gel electrophoresis before beginning library preparation. Additionally, conditions for shearing gDNA to a size that can support producing >30 kb libraries must also be determined and verified empirically for each sample.

Overall yields of >30 kb libraries are typically 5-10%. For large genome projects, we recommend starting this procedure with >20 µg of high quality gDNA sample.

Required Materials

Item	Vendor	Part Number
Pulsed Field Gel Electrophoresis		
Pulsed Field Gel Electrophoresis System: CHEF Mapper XA	Bio-Rad	170-3670
Pulsed Field Certified Agarose	Bio-Rad	162-0137
CHEF DNA Size Standard 5 kb	Bio-Rad	170-3624
Invitrogen 1 kb DNA extension ladder	Life Technologies	10511-012
Shearing		
26G Blunt End Needles	SAI Infusion	B26-150
1 mL Luer-Lok Tip Syringe	Becton Dickinson	309628
SMRTbell Library		
SMRTbell Template Prep Kit 1.0	Pacific Biosciences	100-259-100
SMRTbell Damage Repair Kit	Pacific Biosciences	100-465-900
AMPure PB Beads	Pacific Biosciences	100-265-900
Tube rotator (or equivalent)	VWR	10136-084
Qubit 3.0 Fluorometer and	Life Technologies	Q33216
dsDNA HS Assay Kit or equivalent	Life Technologies	Q32854
Size Selection		
BluePippin Size-Selection System	Sage Science	BLU0001
BluePippin Gel Cassettes	Sage Science	PAC30KB

Workflow

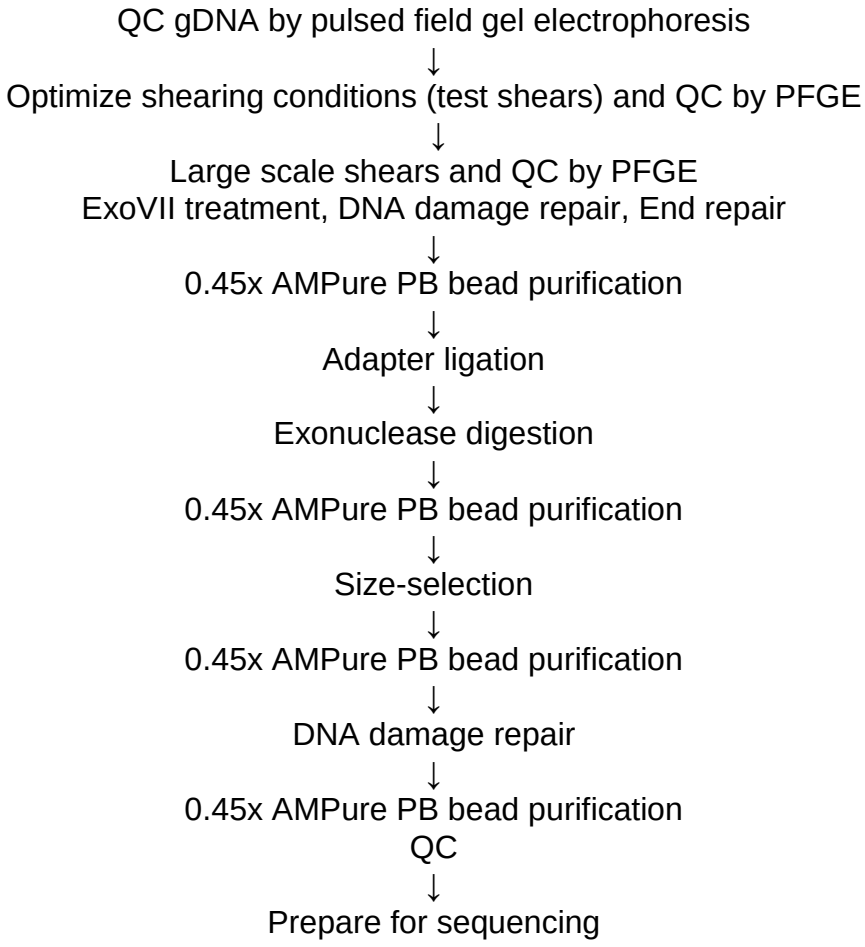
QC gDNA by pulsed field gel electrophoresis
Optimize shearing conditions (test shears) and QC by PFGE
Large scale shears and QC by PFGE
ExoVII treatment, DNA damage repair, End repair
0.45x AMPure PB bead purification
Adapter ligation
Exonuclease digestion
0.45x AMPure PB bead purification
Size-selection
0.45x AMPure PB bead purification
DNA damage repair
0.45x AMPure PB bead purification
QC
Prepare for sequencing

Figure 1: Workflow for preparing >30 kb SMRTbell libraries.

Evaluate Genomic DNA Quality

We highly recommend using the Bio-Rad CHEF Mapper XA Pulsed Field Electrophoresis for resolution above 50 kb. Other systems do not provide good resolution above 50 kb. Genomic DNA suitable for preparing >30-kb libraries will migrate almost exclusively above ~50 kb (for example, see Figure 2, Sample 1). If significant portion of the gDNA migrates below ~50 kb (for example, see Figure 2, Sample 2), do not proceed with the 30 kb size selection protocol. Instead, refer to "Shared Protocol - 20 kb Template Preparation Using BluePippin Size-Selection System (15 kb Size Cutoff)" for recommendations on how to prepare >6-kb to >15-kb libraries.

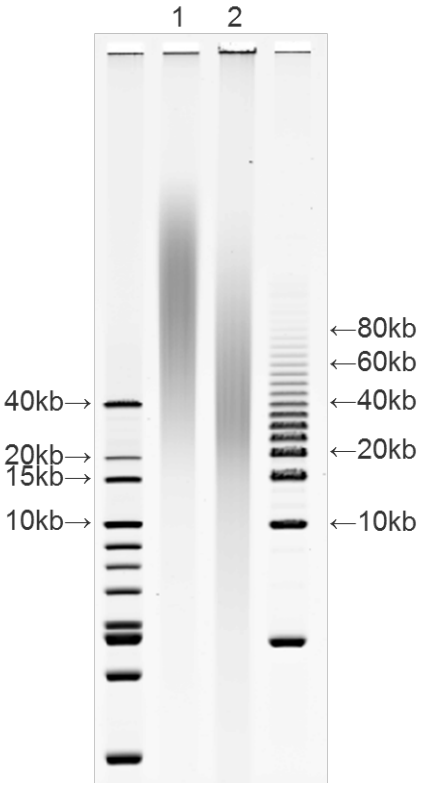

Figure 2: Evaluation of gDNA quality by pulsed-field gel electrophoresis. Lane 1 contains the 1 kb extension DNA ladder, and Lane 4 the Bio-Rad 5 kb DNA ladder. Sample 1 provides an example of high quality, high molecular weight gDNA. The gDNA of Sample 2 should not be used for the production of a >30-kb library.

Optimize Shearing Conditions and Shear gDNA

To ensure sufficient yields of final >30 kb libraries, input gDNA must be sheared carefully so that the average size of fragmented DNA remains well above the desired size-selection cut-off. The response of individual gDNA samples to recommended shearing parameters may differ and must be determined empirically and evaluated by pulsed field gel electrophoresis. Test shears are highly recommended.

Note that for preparing >30 kb libraries, gDNA may be sheared by using a 26G needle. Here we provide initial starting parameters methods as well as strategies for optimization of gDNA shearing.

Shearing using 26G Needles

Before performing needle shearing, please view a short movie demonstrating how to shear using needles https://s3.amazonaws.com/files.pacb.com/mp4/needle-shearing.mp4

Adjust the gDNA concentration to approximately 250 ng/µL with Elution Buffer (EB). If initial DNA concentration is less than 250 ng/µL, concentrate gDNA using AMPure PB Beads as described on page 6.

1. Perform test shears by preparing a 50 µl volume sample in a 1.5 ml LoBind tube. Remove a 1 µL aliquot (un-sheared sample) for use as control when run on pulsed-field gel electrophoresis gel.
2. Aspirate the entire volume and pass sample through a 26G needle five times, then remove a second 1 µL aliquot (5x sample).
3. Pass the sample through the needle five more times and remove a third 1 µL aliquot (10x sample).
4. Finally, pass the sample through the needle ten more times and remove a fourth 1 µL aliquot (20x sample).
5. Evaluate the distribution of the resulting sheared gDNA by running the un-sheared, 5x, 10x, and 20x samples on a pulsed-field gel as described above.

Typical results are shown in Figure 3 for a bacterial gDNA sample sheared as described. Both the 5x and 20x shears shown here were used to prepare >30 kb libraries, with good overall yields and sequencing performance.

If the gDNA sample appears under-sheared, decrease DNA concentration (for example, try 125 ng/µL) and/or increase the number of passes through the needle until you achieve a similar distribution of fragmented gDNA.

If the gDNA sample is over-sheared, reduce the number of passes through the needle (e.g., try 1x and 2x).

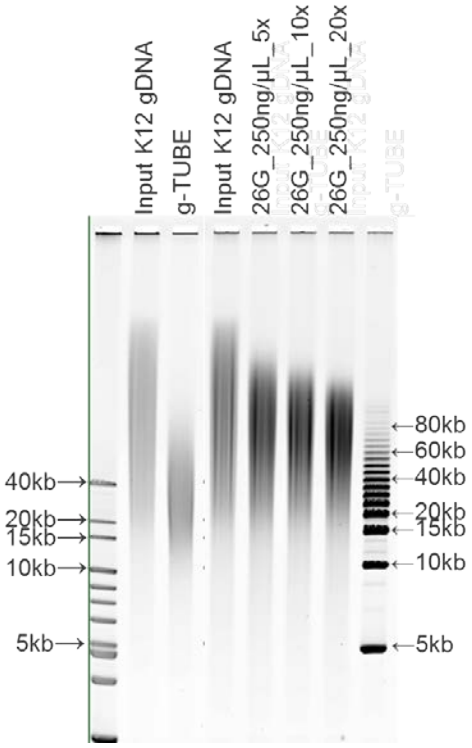

Figure 3: Evaluation of gDNA shears produced by 26G needles using pulsed-field gel electrophoresis. The first lane contains the 1 kb extension DNA ladder, and the last the Bio-Rad 5 kb DNA ladder. Input K12 gDNA=E.coli genomic DNA; g-TUBE=gDNA sheared with g-TUBE; 26G_250ng/ul_5x=5 cycles needle shearing; 26G_250ng/ul_10x=10 cycles needle shearing; 26G_250ng/ul_20x=20 cycles needle shearing.

Large Scale Shearing

Once you have determined the optimal shearing condition, scale up the shearing process by increasing the volume while maintaining the same concentration used during test shears.

Concentrate DNA Using AMPure PB Beads (if necessary)

If the concentration of sheared gDNA is less than 140 ng/µL, concentrate the sheared gDNA using AMPure PB Bead purification before proceeding. If the sheared gDNA concentration is greater than 140 ng/uL, adjust gDNA concentration to 140 ng/µL with EB and proceed directly to ExoVII treatment below.

STEP	✓	Concentrate DNA	Notes
1		Add **0.45X** volume of AMPure® PB magnetic beads. _____µL of sample X **0.45X** = _____µL of beads Note that the beads must be brought to room temperature and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Mix bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by gentle end-over-end rotation for 20 minutes at room temperature. We recommend using a VWR tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this Procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on mmagnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	

STEP	✓	Concentrate DNA	Notes
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 60 seconds.	
13		Calculate appropriate volume of Elution Buffer. ____ng X **0.5** / (____ng/µL) = ____µL of Elution Buffer needed The minimum DNA concentration required to proceed to the next step (End-Repair) is **140 ng/µL** with preferred mass of at least **5 µg**.	
14		Add the Pacific Biosciences® Elution Buffer volume (calculated in step 13) to your beads. Tap the tube with finger gently to mix. Do not pipet to mix. – Elute the DNA by gentle letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Perform concentration measurements. Verify your DNA concentration using a Nanodrop® or Qubit® quantitation platform. If the DNA concentration is estimated to be equal to or below **12 ng/µL**, a Qubit system reading is required. When performing a Qubit system reading, ensure that your sample is within the range of the Qubit kit you are using. For proper concentration calculations, incorporate the dilution factor (used when diluting your sample) to be within range of the Qubit kit and the dilution factor when diluting your sample with the working solution. The latter part of this dilution factor can be calculated automatically by the Qubit system. – Discard the beads.	
15		Perform concentration measurements. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations. Yield up to this point should be 40-60%. The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed field gel electrophoresis (see example in Figure 4).	
16		The sheared DNA can be stored for up to 24 hours at 4°C or at -20°C for longer duration.	
17		Actual recovery per µL and total available sample material: ___	

ExoVII Pre-treatment of DNA

Use the following table to set up a reaction to remove single-stranded ends from 5 µg of sheared gDNA at 140 ng/µL. If starting with more than 5 µg of sheared gDNA, scale reaction volumes proportionally (i.e., for 10 µg of DNA scale the total volume to 96 µL).

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.		Notes
Sheared DNA (5 µg)	–		36.0 µL	–		
DNA Damage Repair Buffer		10 X	5.0 µL	1 X		
NAD+		100 X	0.5 µL	1 X		
ATP high		10 mM	5.0 µL	1 mM		
dNTP		10 mM	0.5 µL	0.1 mM		
ExoVII		10 U/µL	1.0 µL	0.2 U/µL		
Total Volume			48.0 µL	–		

2. Mix the reaction well by gently tapping the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 37°C for 15 minutes, then return the reaction to 4°C.

Repair DNA Damage

Use the following table to prepare your reaction. For more than 5 µg input DNA, scale all reaction volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (ExoVII treated)	–		48 µL	–		
DNA Damage Repair Mix		25 X	2.0 µL	1X		
Total Volume			50.0 µL	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 60 minutes, return the reaction to 4°C for 1 to 5 minutes.

Repair Ends

Use the following table to prepare your reaction. For more than 5 µg input DNA, scale all reaction volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (Damage Repaired)	–		52.0 µL	–		
End Repair Mix		20 X	2.5 µL	1X		
Total Volume			52.5 µL	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25°C for 5-10 minutes, return the reaction to 4°C.

Purify DNA Using 0.45X AMPure PB Beads

STEP	✓	Purify DNA	Notes
1		Add **0.45X** volume of AMPure PB beads to the End-Repair reaction.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 20 minutes at room temperature. We recommend using a VWR tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 60 seconds.	
13		For 5 µg input sheared gDNA, elute in 23 µL Elution buffer. If you started with more than 5 µg input sheared gDNA, scale volume of EB proportionally (i.e., for 10 µg of DNA, elute in 46 µL EB). Mix by gently tapping the tube. Elute by letting it sit at room temperature for 2 minutes.	
14		Optional: Verify your DNA amount and concentration using a Qubit quantitation platform.	
15		The End-Repaired DNA can be stored overnight at 4°C or at -20°C for longer durations.	
16		Actual recovery per µL and total available sample material: _____	

End-Repaired insert DNA can be stored overnight at 4°C or at -20°C for longer durations.

Prepare Blunt-Ligation Reaction

Use the following table to prepare your reaction, adding the components below in the order listed. Be sure to mix insert gDNA and adapter BEFORE adding ligase. For more than 5 µg input DNA, scale all reaction volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)	–		23.0 µL			
Annealed Blunt Adapter (20 µM)		20 µM	10 µL	5 µM		
Finger tap to mix before proceeding						
Template Prep Buffer		10 X	4.0 µL	1X		
ATP low		1 mM	2.0 µL	0.05 mM		
Finger tap to mix before proceeding						
Ligase		30 U/µL	1.0 µL	0.75 U/µL		
Total Volume	–	–	40.0 µL	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25°C or room temperature overnight.
4. Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C.

ExoIII/VII Digestion to Remove Failed Ligation Products

Use the following table to prepare your reaction. For more than 5 µg input DNA,scale all reaction volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume
Ligated DNA			40 µL
ExoIII		100.0 U/µL	1.0 µL
ExoVII		10.0 U/µL	1.0 µL
Total Volume			42 µL

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge
3. Incubate at 37°C for 1 hour, then return the reaction to 4°C. You must immediately proceed with AMPure PB bead purification after this step.

Purify SMRTbell™ Templates with 0.45X AMPure PB Beads

STEP	✓	Purify DNA	Notes
1		Add **0.45X** volume of AMPure PB beads to the End-Repair reaction.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 20 minutes at room temperature. We recommend using a VWR tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 60 seconds.	
13		For up to 10 µg input sheared gDNA, elute in 31 µL Elution buffer. If you started with more than 5 µg input sheared gDNA, scale volume of EB proportionally (i.e., for 20 µg of DNA, elute in 62 µL EB). Mix by gently tapping the tube. Elute by letting it sit at room temperature for 2 minutes.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations. Yield up to this point should be 40-60%. The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed field gel electrophoresis (see example in Figure 4).	
15		Optional: Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit. Note that typical yield at this point of the process (following End-Repair and one **0.45X** AMPure PB bead purification) is approximately between **80-100%** of the total starting material.	
16		Actual recovery per µL and total available sample material: _____	

Proceed immediately with size-selection after AMPure PB Bead purification of exonuclease-treated libraries. Samples may be stored at -20°C for short periods at this stage, if necessary.

Size-Selection Using the BluePippin™ System

Follow the instructions in the BluePippin User Manual and User Guides (see www.sagescience.com), and the specific recommendations below, for >30 kb or 40 kb size-selection of the SMRTbell templates.

Note that you must use BluePippin Software **v6.20** and the "0.75%, DF Marker U1 high-pass 30 kb – 40 kb v3" run protocol for this procedure. A new size standard is required for this protocol. Use the U1 marker.

1. Prepare up to 5 µg SMRTbell templates in a final volume of 30 µL Elution Buffer for each lane. Size selection using this protocol can be aggressive and if not careful, recovery may be impacted. It is not recommended to use less than 4 µg per lane.

2. Bring the Loading Solution to room temperature, then add 10 µL of the Loading Solution to the 30 µL DNA sample. For multiple lanes, scale volumes proportionally. The Loading Solution is viscous so pipet slowly to ensure complete transfer into the DNA sample.
    a. Mix by tapping the tube gently; do not vortex.
    b. Spin briefly to collect the contents at the bottom of the tube.

3. Follow the manufacturer's recommendations to set up a run protocol.
    a. When setting up the run protocol, select the "0.75% DF marker S1 high-pass 30-40kb v3" cassette definition file.
    b. Using the "Range" selection mode, enter the desired "BPstart" value of 30000 or 40000 bp. A "BP End" value of 80000 bp should automatically appear. Be sure to assign a marker lane.

4. Load samples and start the run. Be sure to include the U1 marker in the correct lane. Typical run times are ~10 hours.

5. To maximize recovery of eluted DNA, wait at least 45 minutes after the run terminates before removing the sample from the elution chamber.
    a. Collect the eluate into a 1.5 mL DNA LoBind tube.
    b. Wash elution well with 40 µL of Sage Science's 0.1% Tween-20 Wash Solution, and add wash to eluted sample. Washing the elution well may increase yield 10-20%.

Purify Size-Selected SMRTbell™ Templates with 0.45X AMPure PB Beads

STEP	✓	Purify DNA	Notes
1		Add **0.45X** volume of AMPure PB beads to the End-Repair reaction.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 20 minutes at room temperature. We recommend using a VWR tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 60 seconds.	
13		For up to 5 µg input non-size-selected library gDNA, elute in 23 µL Elution buffer. If you size-selected more than 5 µg of SMRTbell template, scale volume of EB proportionally (i.e., for up to 10 µg of input DNA, elute in 20 µL EB). Mix by gently tapping the tube, elute by letting the tube sit for two minutes at room temperature.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Using 1 µL of the purified sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations. Size-selection set yield should be 20-40%. The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed field gel electrophoresis.	
15		Actual recovery per µL and total available sample material: _____	

AMPure PB bead purified, size-selected libraries may be stored at -20°C.

Repair DNA Damage After Size-Selection

Using the table below, set up a reaction to repair any DNA damage present in SMRTbell templates after >30 kb or >40 kb size-selection. For up to 5 µg of DNA, use a reaction volume of 50 µL. If starting with more than 5 µg of size-selected template, scale reaction volumes proportionally (i.e., for up to 10 µg of DNA use a 100 µL reaction volume).

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.		Notes
Size-selected DNA	–		__ µL for 5.0 µg	–		
DNA Damage Repair Buffer		10 X	5.0 µL	1 X		
NAD+		100 X	0.5 µL	1 X		
ATP high		10 mM	5.0 µL	1 mM		
dNTP		10 mM	0.5 µL	0.1 mM		
DNA Damage Repair Mix		25 X	2.0 µL	1X		
H_2O	–		__ µL to adjust to 50.0 µL	–		
Total Volume			50.0 µL	–		

2. Mix the reaction well by gently tapping the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 37°C for 60 minutes.

Purify Damage-Repaired, Size-Selected SMRTbell™ Templates with 0.45X AMPure PB Beads

STEP	✓	Purify DNA	Notes
1		Add **0.45X** volume of AMPure PB beads to the End-Repair reaction.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 20 minutes at room temperature. We recommend using a VWR tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 60 seconds.	
13		For up to 5 µg size-selected library, elute in 10 µL Elution buffer. For more than 5 µg of SMRTbell template, scale volume of EB proportionally (i.e., for up to 10 µg of input DNA, elute in 20 µL EB). Mix by gently tapping the tube, elute by letting it sit for two minutes at room temperature.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Using 1 µL of the purified sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations. The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed field gel electrophoresis (see example in Figure 4).	

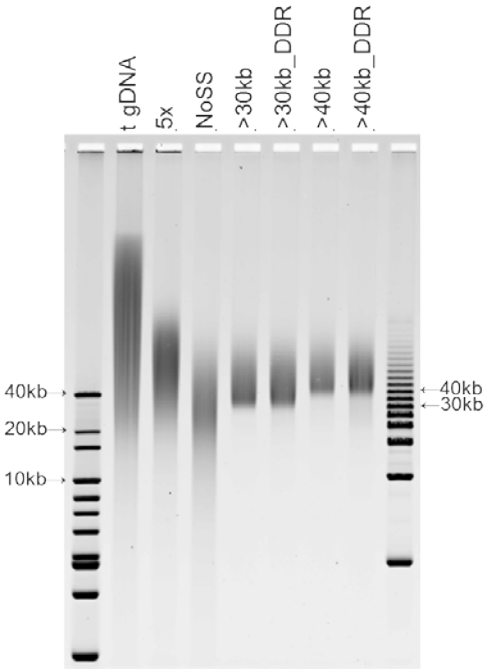

Figure 4: Evaluation of >30 kb and >40 kb libraries using pulsed-field gel electrophoresis. The first lane contains the 1 kb extension DNA ladder, and the last the Bio-Rad 5 kb DNA ladder. Samples are as indicated above each lane. 5x=5 cycles needle shearing NoSS=no size selection, >30kb = 30kb cutoff with no DNA Damage repair treatment after size selection, >30kb_DDR = 30kb cutoff with DNA Damage repair treatment after size selection, >40kb = 40kb cutoff with no DNA Damage repair treatment after size selection, >40kb_DDR = 40kb cutoff with DNA Damage repair treatment after size selection.

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Anneal and Bind BluePippin™ Size-Selected SMRTbell™ Templates

Using the Binding Calculator, compute the molarity of your library by using an average insert size of 30000 bp for >30 kb libraries, and 40000 bp for >40 kb libraries.

Anneal 20X sequencing primer at a template concentration of 0.833 nM, as directed by the Binding Calculator. Before adding the primer to the SMRTbell template, pre-condition the primer by heating to 80ºC for 2 minutes, then placing immediately on ice. (Note that if kept on ice during use, and stored at -20ºC, pre-conditioned primer may be used multiple times without re-heating.) Add the appropriate amounts of pre-conditioned primer and 10X Primer Buffer to the SMRTbell template, and incubate at 20ºC for at least 30 minutes.

Bind 10X P6 polymerase at an annealed template concentration of 0.500 nM, according to the Binding Calculator. For >30 and >40 kb libraries, incubation of the binding reaction at 30ºC for 4 hours may modestly improve loading relative to 30 minute binding reactions.

Prepare for MagBead Loading

Optimal loading of >30 and >40 kb SMRTbell libraries using P6 polymerase can typically be achieved using an on-plate concentration of ~0.225nM to 0.375 nM. We recommend you perform an initial loading titration in this range to determine optimal loading for your sample, for example 0.225 nM, 0.300 nM and 0.375 nM.

For efficient binding to Magnetic Beads, bound complexes (at 0.500 nM concentration) must be diluted in the appropriate ratio of MagBead Binding Buffer and MagBead Wash Buffer. Follow the Binding Calculator instructions to dilute your sample for MagBead binding.

SMRT® Cells for Large-Scale Sequencing Projects

A loading titration should be performed with each new SMRT Cell lot to determine the optimal on-plate sample concentrations. If possible, large multi-SMRT Cell genome projects should be completed with a minimum number of lots to decrease the number of loading titrations. When placing an order of SMRT Cells, indicate on the Purchase Order to '*MINIMIZE SMRT Cell lots'*. Please use this exact phrase.

Control Complex Dilution

If you will be using the PacBio® Control Complex, dilute the DNA Control Complex according to the volumes and instructions specified in the Calculator.

Sequence

When sequencing >30 and >40 kb SMRTbell libraries, be sure to choose the correct magnetic bead collection protocol (MagBead Standard Seq v2 or MagBead OneCellPerWell v1), indicate a 20,000 bp insert size, and select stage start when setting up your run protocol. We recommend 240 or 360 minute movies for >30 and >40 kb libraries.

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